



Chemical profiling and quantification of monacolins and citrinin in red yeast rice commercial raw materials and dietary supplements using liquid chromatography-accurate QToF mass spectrometry: Chemometrics application



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ABSTRACT

Red yeast rice (RYR) is prepared by fermenting rice with various strains of the yeast *Monascus* spp of the Aspergillaceae family. Depending on the *Monascus* strains and the fermentation conditions, the products may contain monacolins, pigments and citrinin as secondary metabolites. Authentic and commercial RYR samples were analyzed using UHPLC-DAD-QToF-MS for monacolins, pigments and citrinin. A separation by UHPLC was achieved using a reversed-phase column and a gradient of water/acetonitrile each containing formic acid as the mobile phase. Accurate mass QToF spectrometry was used to distinguish isobaric monacolins. Principle component analysis (PCA), a chemometric technique was used to discriminate between authentic RYR, commercial RYR raw materials and dietary supplements. Three authentic RYR samples, 31 commercial RYR raw materials and 14 RYR dietary supplements were analyzed. Monacolin K content in 600 mg of authentic RYR samples ranged from 1.2 mg to 1.38 mg. Amounts of monacolin K in dietary supplements labeled as containing 600 mg of RYR varied more than 40-fold from 0.03 mg to 2.18 mg. Monacolin K content of dietary supplements labeled as containing 1200 mg RYR varied more than 20-fold from 0.22 mg to 5.23 mg. In addition to large variations in quantity of monacolin K found in dietary supplements, RYR dietary supplements contained ratios of monacolins that differed significantly from authentic samples. The results indicated that RYR commercial products are of variable quality and the analytical method is suitable for quality control testing of a variety of RYR products.

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1. Introduction

Red yeast rice (RYR) is rice that has been fermented with the mold *Monascus purpureus* [1]. RYR contains a pharmacologically important group of compounds belonging to the polyketides-monacolins J, K (lovastatin), L, M and X as well as acid forms of J, K, L, M and X, and dihydromonacolin K. Among these, monacolin

K and its dihydro derivatives are the most active compounds [2–4]. Red yeast rice has been used for centuries as a food as well as a traditional Chinese medicine which the Pharmacopeia of the People's Republic of China describes as invigorating the body, aiding in digestion, and revitalizing the blood [1]. More recently, RYR has been used in the treatment of hypercholesterolemia [3–5]. Monacolin K (also known as lovastatin or mevinolin) and its hydroxy acid form the main monacolins in *Monascus purpureus*-fermented rice (75–90% of total monacolin content) [6,7]. The monacolin K content of one proprietary preparation of red yeast rice used in a clinical trial was calculated to be 0.2% of the total product which provided a daily dose of 4.8 mg of monacolin K (in 2.4 g of red yeast rice) [6,8]. This trial found the standardized RYR product effective in

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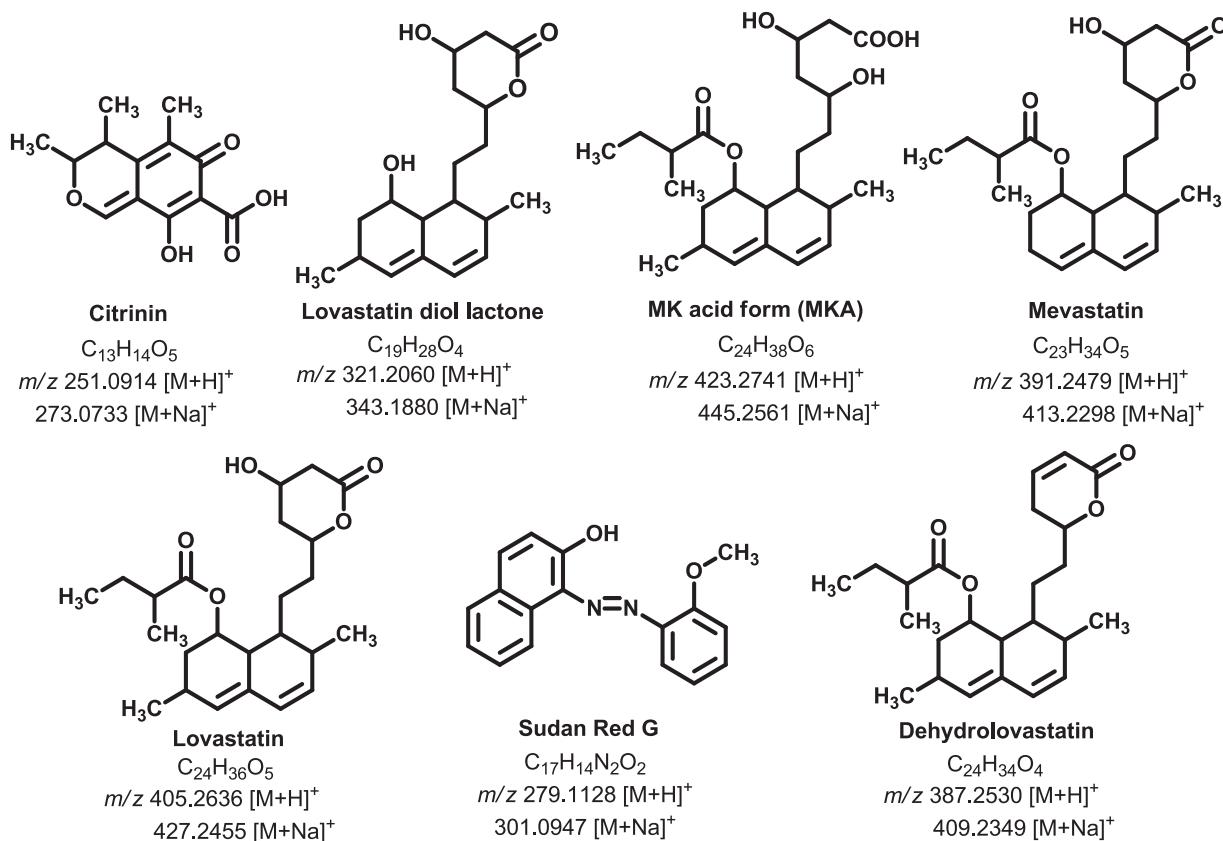


Fig. 1. Structure of reference compounds.

lowering cholesterol levels; however, there has been concern raised about the common practice of using other red yeast rice products to treat hypercholesterolemia because of wide variability of monacolin K and other active compounds in commercially available RYR products [7,9].

In addition to the monacolins, pigments produced by the mold *Monascus* spp. have a long history of use in Asia as coloring agents. These pigments are very stable compared to synthetic dyes. The pigments of *Monascus* improve the appearance of foods [1]. These include the orange pigments monascorubin (MS) and rubropunctatin, the yellow pigments monascin and ankaflavin (AK), and the red pigments monascorubramine and rubropunctamine. The color of these pigments is influenced by the culture conditions, in particular by the pH and the phosphorus and nitrogen source in the substrate [2,10,11].

Analytical methods [5,7,12–20] have been reported for the determination of individual monacolin K or monacolin content and/or citrinin using liquid chromatography [5,7,12–19] and capillary electrophoresis [20]. Most commonly, high-performance liquid chromatography (HPLC) with diode array detection (DAD) and/or with mass spectrometry were reported for the determination of monacolins or monacolin K and citrinin in red yeast rice and/or commercial products. In these methods [5,7,12–14], the run time per sample took about 25–40 min. The detection limits for monacolin K was found to be in the range from 0.2 to 0.5 μ g/ml. The injection volumes were between 5 and 20 μ l and only few validation parameters were tested. In other methods, screening of products for adulteration [15], stability stress test [16] of one RYR sample, cytotoxicity screening [17], and extraction parameters [18] were studied. Wang et al., [19] showed the fragmentation studies of lovastatin and simvastatin using tandem mass spectrometry. Biljana et al., [20] described analysis of lovastatin and citrinin using

capillary electrophoresis. In all these methods, the samples used were very few in number.

The method described herein is highly sensitive and suitable for qualitative analysis of wide range of compounds including 12 monacolins, 13 pigments and citrinin. Testing for sudan red G (dye) was also performed because sudan red G can be found as an adulterant in RYR [21]. Quantification (monacolins, citrinin and sudan red G) and validation was achieved by this method (structures shown in Fig. 1) in a single run. This developed method offered smaller sample volume/solvent requirement, shorter run time and high resolution. The ability to distinguish between various RYR samples (34 RYR raw materials and 14 commercial products) using fingerprinting technique and chemometrics (PCA) could be a powerful tool to assure the identity and quality of the botanical raw materials and to support the safety and efficacy of the botanical products.

2. Experimental

2.1. Chemicals and plant materials

2.1.1. Chemicals

Citrinin (**1**), lovastatin (monacolin K) (**5**) and sudan red G (**6**) were purchased from Sigma (St. Louis, MO, USA). Mevastatin (compactin) (**4**) was obtained from Chromadex (Santa Ana, CA, USA). Lovastatin diol lactone (monacolin J) (**2**) was purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Dehydrolovastatin (dehydromonacolin K) (**7**) was obtained from Toronto Research Chemicals Inc., North York, ON, Canada. Monacolin K acid (Lovastatin acid) (**3**) was prepared by hydrolyzing lovastatin with a methanolic 0.1 N sodium hydroxide solution under optimized conditions as described in literature [7,17,18] and confirmed using LC-QToF-MS analysis. The analysis confirmed that the lactone form

was completely converted to the acid form. The identity and purity of these compounds were confirmed by chromatographic (HPLC) and spectrophotometric techniques (ESI-HRMS) and comparison with published spectral data. The purity of these compounds was greater than 97%. Acetonitrile and formic acid were of HPLC grade purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water for the HPLC mobile phase was purified using a Milli-Q system (Millipore).

2.1.2. RYR samples

Three authentic samples (# AU15680–AU15682) were obtained from Beijing Peking University WBL Biotech Co., Ltd., China. Thirty-one samples of red yeast rice raw materials (#CR15674–CR15685, CR15688–CR15691, CR15693–CR15695, CR15696–CR15706, CR15708–CR15711) were procured from China. These are the raw materials frequently purchased by supplement manufacturers to produce RYR dietary supplements in USA and elsewhere. In addition, 14 dietary supplements (#DS15715–DS15728) labeled as containing 600 or 1200 mg of RYR were purchased online from supplement retailers in USA. Specimens of all samples are deposited at the NCNPR's botanical repository, The University of Mississippi, University, MS, USA.

2.1.3. Preparation of standard solutions

Stock solutions of the standard compounds were prepared at a concentration of 2 mg/ml in methanol. Calibration curves were prepared at seven different concentration levels. The concentrations range were 0.5–100 µg/ml for compound **1** and 0.3–500 µg/ml for compounds **2–7** using a UHPLC–DAD method.

2.1.4. Sample preparation

2.1.4.1. Capsules/tablets. Five tablets were weighed and then pulverized with a mortar and pestle. For capsules, five samples were weighed, opened and the contents mixed and triturated in a mortar and pestle.

2.1.4.2. Solids. Dry plant samples (0.5 g) or an adequate amount of capsules content or tablets were weighed (average weight of dosage form) then sonicated in 2.5 ml of methanol for 30 min followed by centrifugation for 15 min at 959 × g. The supernatant was transferred to a 10 ml volumetric flask. The procedure was repeated four more times with 2.0 ml methanol and the respective supernatants were combined. The final volume was adjusted to 10 ml with methanol and mixed thoroughly. Prior to injection, an adequate volume (*ca.* 2 ml) was passed through a 0.45 µm PTFE membrane filter. The first 1.0 ml was discarded and the remaining liquid was collected in an LC sample vial.

2.2. Instrumentation and chromatographic conditions

2.2.1. Ultra-high performance liquid chromatography–Diode array detector–Mass spectrometry (UHPLC–DAD–QToF–MS)

The liquid chromatographic system was an Agilent Series 1290 comprised of the following modular components: a binary pump, vacuum solvent microdegasser, autosampler with 108-well tray, thermostatically controlled column compartment, and photo diode array detector. Separation was achieved on an Agilent Zorbax SB-C18 RRHD (2.1 × 150 mm, 1.8 µm) column. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at a flow rate of 0.35 ml/min. Analysis was performed using the following gradient elution: 65% A/35% B–35% A/65% B in 15 min and in next 3 min to 100% B. Each run was followed by a 5-min wash with 100% B and an equilibration period of 5 min with 65% A/35% B. Each run was followed by a 5 min wash with 100% B and an equilibration period of 5 min with 65% A/35% B. Two microliters of sample was injected. The column temperature was 35 °C.

The mass spectrometric analysis was performed with a QToF-MS–MS (Model #G6530A, Agilent Technologies, Santa Clara, CA, USA) equipped with an ESI source with Jet Stream technology using the following parameters: drying gas (N₂) flow rate, 9.0 L/min; drying gas temperature, 250 °C; nebulizer pressure, 35 psig, sheath gas temperature, 325 °C; sheath gas flow, 10 L/min; capillary voltage, 3500 V; nozzle voltage, 0 V; skimmer, 65 V; Oct RF V, 750 V; and fragmentor voltage, 125 V. All the operations, acquisition and analysis of data were controlled by Agilent MassHunter Acquisition Software Ver. A.05.01 and processed with MassHunter Qualitative Analysis software Ver. B.06.00. Each sample was analyzed in positive mode over the range of *m/z* = 100–1000 and extended dynamic range (flight time to *m/z* 1700 at 2 GHz acquisition rate). Accurate mass measurements were obtained by means of reference ion correction using reference masses at *m/z* 121.0509 (protonated purine) and 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine or HP-921] in positive ion mode. The compounds were confirmed in each spectrum. For this purpose, the reference solution was introduced into the ESI source via a T-junction using an Agilent Series 1200 isocratic pump (Agilent Technologies, Santa Clara, CA, USA) using a 100:1 splitter set at a flow rate of 20 µl/min.

For recording ToF-MS spectra, the quadrupole was set to pass all ions (RF only mode) and all ions were transmitted into the pusher region of the time-of-flight analyzer where they were mass-analyzed with a 1-s integration time. For the ESI-MS–MS CID experiments, precursor ions of interest were mass-selected by the quadrupole mass filter. The selected ions were then subjected to collision with nitrogen in a high pressure collision cell. The collision energy was optimized to yield good product ion signals, which were subsequently analyzed with the ToF mass spectrometer. Analysis was performed in the reflectron mode with a resolving power of about 10,000 at *m/z* 922.0098. The instrument was set to extended dynamic range (up to 10⁵ with lower resolving power). MS–MS spectra were recorded simultaneously at a rate 2.0 spectra s⁻¹. In order to filter selected precursor ions for MS–MS, an isolation window of 4.0 *m/z* was set for the quadrupole. Tandem mass spectrometry (MS–MS) studies were performed by isolating [M+H]⁺ ions. The major fragments were obtained for the compounds of monacolins, pigments, citrinin and sudan red G by means of collision induced dissociation (CID) of the protonated molecule and compared with the similar data produced for standard compounds under the same conditions.

2.2.2. Data processing

MassHunter Workstation software, including Qualitative Analysis (version B.06.00), was used for processing both raw MS and MS–MS data, including molecular feature extraction, background subtraction, data filtering, and molecular formula estimation. To perform subtraction of molecular features (MFs) originating from the background, analysis of a blank sample (methanol) was carried out under identical instrument settings and background MFs were removed. MFs were characterized by retention time, intensity at the apex of the chromatographic peak and accurate mass. Background subtracted data were converted into compound exchange format (cef) files for further use in Mass Profiler Professional (MPP). MPP (Agilent, version 12.6) was used for statistical evaluation of technical reproducibility and comparison of RYR samples and dietary supplements. In MPP, the retention time and *m/z* alignment across the sample sets was performed using a tolerance window of 0.2 min and 20 mDa.

2.3. Validation procedure

The newly developed UHPLC method was validated in terms of system suitability, accuracy, precision, LOD, LOQ, linearity, range,

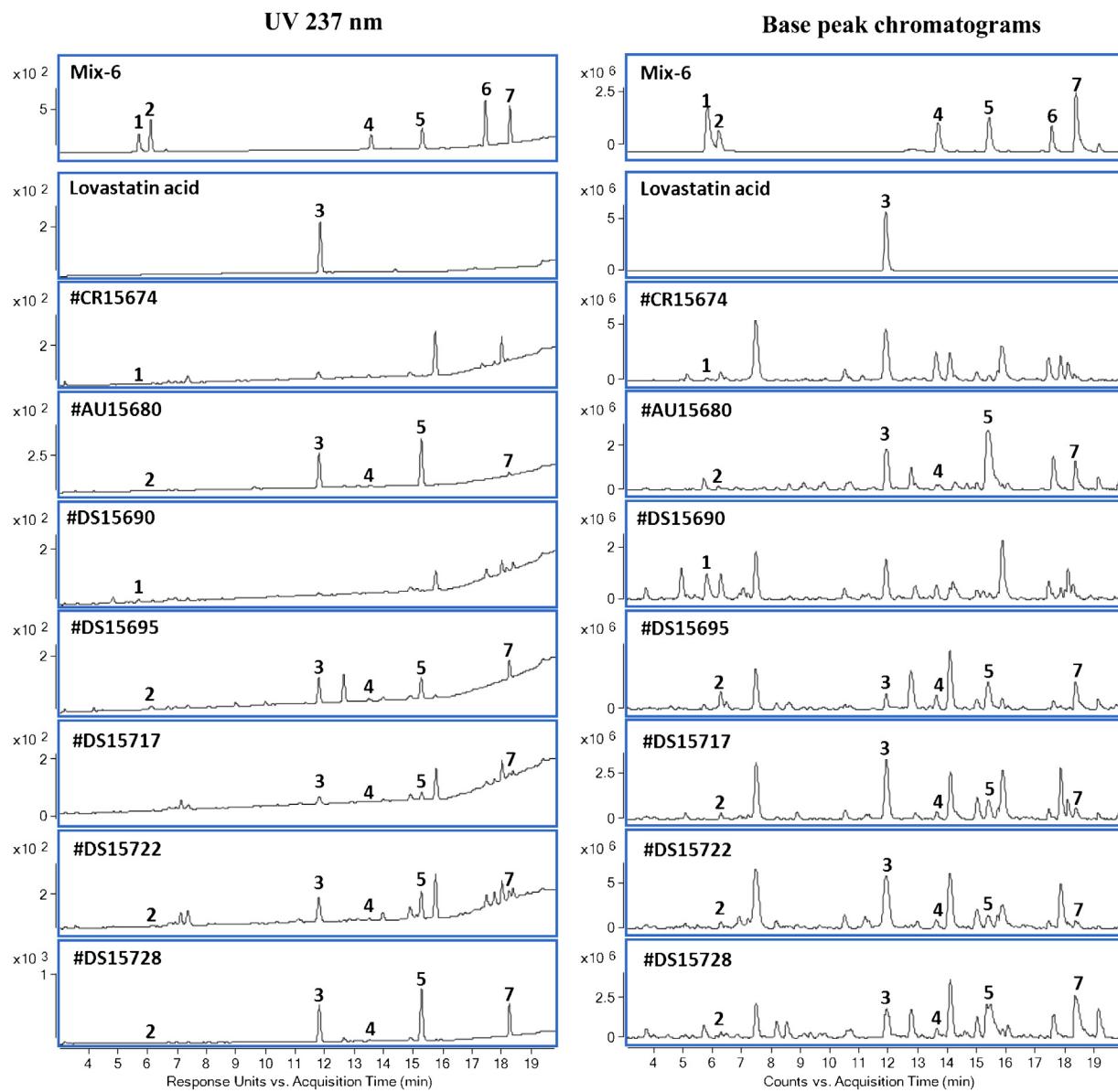


Fig. 2. UHPLC-UV chromatograms and base peak chromatograms of the RYR samples including dietary supplements analyzed using UHPLC-DAD-QToF-MS in positive ESI mode. Column, Zorbax SB-C18 RRHD (2.1 × 150 mm, 1.8 μm); mobile phase, water and acetonitrile both containing formic acid, gradient; 35 °C; UV, 237 nm and ESI +ve mode.

selectivity, stability, matrix effects and robustness according to ICH guidelines [22]. Limits of detection (LOD) and limit of quantification (LOQ) were determined by injecting a series of dilute solutions with known concentrations for each standard. LOD and LOQ were defined as the signal-to-noise ratio equal to 3:1 and 10:1, respectively. The accuracy of the assay method was evaluated in triplicate using two different levels 50% and 100%. Intra- and inter-day variation of the assay was determined on 3 consecutive days with 3 repetitions each. Robustness was carried out during the development stage of the method. Small deliberate changes to the procedure and their results were assessed. System suitability tests were used to ensure reproducibility of the instrument. The test was carried out by injecting 2 μl of mixture of standard solution at least six times. The sample solution and standard solution were prepared by the proposed method and subjected to a stability study at room temperature for 24 h.

3. Results and discussion

3.1. Optimization of conditions for UHPLC-QToF-MS

3.1.1. Method development

In the present study, targeted analysis of reference compounds and auto MS-MS analysis of RYR methanol extracts and dietary supplements were performed using a reversed-phase chromatographic system without considering any specific group of compounds. UHPLC-QToF-MS conditions were optimized to detect the maximum number of compounds including reference standards. The concentrations of sample solutions were optimized to 50 mg/ml. Q-ToF provides accurate MS-MS spectra using internal mass calibration during acquisition and mass drift compensation (collected in single MS mode and applied to the MS-MS as a rolling average). Mass accuracy of better than 2 ppm was obtained. Fig. 2

represents the DAD (237 nm) and ESI-MS base peak chromatograms (BPC) of a methanolic extract of commercial RYR samples and dietary supplements. The solvent system was optimized to elute all the detectable compounds within 20 min. The elution was continued for an additional 5 min to ensure the complete removal of sample from the column. Retention time variability across the samples was found to be 3–4 s with a relative standard deviation of less than 2%.

3.1.2. Extraction

The RYR powder was a complex mixture of compounds with a wide range of polarities. Two extraction techniques were employed. Firstly authenticated RYR powder (#AU15680) was exhaustively treated with methanol as described above under section "Sample Preparation". Secondly the RYR authentic sample (#AU15680) was also exhaustively treated with 75% ethanol. In comparison both methods showed the almost equal amounts of all compounds. Hence all samples extractions were performed with 100% methanol.

3.1.3. Chemical fingerprint analysis

Chemical differences were found between the samples of monacolin derived RYR samples and pigment derived RYR samples. Samples (#CR15674–CR15678, CR15688–CR15691, CR15609) of RYR fermented by *Monascus* spp. had high amounts of pigments and no monacolins, RYR samples and dietary supplements (#AU15680–AU15682, CR15683–CR15687, CR15693–CR15695, CR15797–CR15706, CR15708, CR15710–CR15711, DS15715–DS15725) were found to have monacolins and few pigments. The molecular formulae were deduced and short listed by comparing the accurately measured mass values to the exact mass values of protonated molecules ($[M+H]^+$). Table 1S shows the qualitative analysis of all samples including dietary supplements with respect to 12 monacolins, 13 pigments, citrinin and sudan red G.

The use of QToF for mass accuracy and resolution is important in solving complex analytical problems, and a valuable tool for determination of compound identity. For example, two isobaric compounds showed the same nominal mass of m/z 405 $[M+H]^+$. The formulae of the protonated molecules were found to be $C_{24}H_{36}O_5$ (m/z 405.2636) and $C_{23}H_{32}O_6$ (m/z 405.2272), and the accurate mass aided in identification of lovastatin (monacolin K) which showed m/z 405.2636 not m/z 405.2272. Similarly other compounds having identical nominal masses [$C_{23}H_{34}O_6$ (m/z 406.2355), monacolin M; $C_{24}H_{38}O_5$ (m/z 406.2719), dihydromonacolin K] have been distinguished accordingly using mass accuracy. Further confirmation of compounds identity was carried out based on retention times and fragment pattern relative to the reference compounds. In Table 2, the MS and MS–MS data for compounds 1–7 are listed, in which the proposed molecular formulas and possible compounds were given on the basis of retention time, calculated mass and information from reference standards. Due to the non-availability of some standards the identity was purely based on accurate MS and its fragmentation behavior (Table 1 and Table 2S).

3.2. Identification and confirmation of monacolins citrinin and sudan red G

Under typical ESI-MS conditions, monacolins, citrinin and sudan red G produced abundant ions corresponding to the protonated molecules $[M+H]^+$, sodium ion adducts $[M+Na]^+$ and/or dimer $[2M+Na]^+$. In the positive ion mode with extracted ion chromatogram (EIC) at m/z 251.0913 $[M+H]^+$, 273.0725 $[M+Na]^+$ for compound 1, m/z 321.2059 $[M+H]^+$, 343.1881 $[M+Na]^+$ and 663.3879 $[2M+Na]^+$ for compound 2, m/z 423.2740 $[M+H]^+$, 445.2565 $[M+Na]^+$ and 867.5239 $[2M+Na]^+$ for compound 3, m/z

391.2479 $[M+H]^+$, 413.2304 $[M+Na]^+$ and 803.4710 $[2M+Na]^+$ for compound 4, m/z 405.2639 $[M+H]^+$, 427.2460 $[M+Na]^+$ and 831.5013 $[2M+Na]^+$ for compound 5, m/z 279.1129 $[M+H]^+$ for compound 6 and m/z 387.2529 $[M+H]^+$, 409.2350 $[M+Na]^+$ and 795.4810 $[2M+Na]^+$ for compound 7. Further, the fragmentation patterns observed in the mass spectrum were useful in characterization of the compounds. The elimination of the ester side chain followed by dehydration and dissociation of the lactone moiety were observed as the main fragmentation pathways for most of the monacolin-type of compounds. Monacolin K (m/z 405.2647 $[M+H]^+$, $C_{24}H_{36}O_5$) showed major fragment ions at m/z 285.1823, 225.1631 and 173.1308 and a base peak at m/z 199.1469 were observed. MS spectrum in the positive ion mode revealed a molecular ion $[M+H]^+$ at m/z 251.0913 for citrinin and hence a molecular formula is $C_{13}H_{14}O_5$ and showed fragment ions at m/z 233.0803, 205.0849, 191.0688, 177.0182, 147.0797, 119.0858, and 91.0539. A base peak at m/z 233.0801 was observed from the loss of water for citrinin. Sudan red G dye showed a fragment corresponding to the loss of an OH⁻ at m/z 262.1083. It also showed a base peak at m/z 123.0676 (Table 1). The identities, retention time (RT), $[M+H]^+$, and characteristic fragment ions for individual peaks are presented in Table 1. The other compounds including monacolins and pigments were also observed based on the accurate mass results and reported methods [20] (Table 2S). No interfering peaks were found at the retention times of interest. Further, the major fragment ions observed in the mass spectrum were useful in the compounds confirmation.

3.3. Quantification of five monacolins, citrinin and sudan red G

The quantification of five monacolins (monacolin K, monacolin K acid, monacolin J, compactin, dehydromonacolin K), citrinin and sudan red G were carried out using the UHPLC–UV method at wavelength 237 nm. The UHPLC–UV method described was tested with respect to sensitivity [the limit of detection (LOD) and the limit of quantification (LOQ)], linearity, intra-day and inter-day precision for three consecutive days, accuracy, specificity, stability, system suitability and robustness.

3.3.1. Method validation

The validation study allowed the evaluation of the method for its suitability for routine analysis.

3.3.1.1. Linearity, range, LOD and LOQ. Good linearity was achieved in the concentration ranges of 0.5–100 $\mu\text{g/ml}$ for citrinin and 0.3–500 $\mu\text{g/ml}$ for other reference compounds. The correlation coefficient was $R^2 = 0.999$ for all reference compounds. The experiment was performed three times and the mean was used for the calculations. The signal-to-noise ratio of 3:1 and 10:1 was used to establish LOD and LOQ, respectively. The LOD and LOQ of citrinin were 0.2 $\mu\text{g/ml}$ and 0.5 $\mu\text{g/ml}$ and for all other 6 compounds were 0.1 $\mu\text{g/ml}$ and 0.3 $\mu\text{g/ml}$, respectively.

3.3.1.2. Specificity and matrix effect. Injection of a RYR powder (#CR15674), consisting of all matrix components with the exception monacolin compounds, in duplicate under the conditions of the developed UHPLC method showed that there was no interference from other components in the sample matrix. In addition, peak homogeneity and peak purity test (DAD, MS) showed that the peak was due to only to one component with peak-purity coefficient of 0.999. So the method was considered to be specific for monacolins analysis.

3.3.1.3. Precision. Intra- and inter-day variation of the analysis was determined for authentic sample # AS15680 and was less than 5%, with a maximum RSD of 2.7%. The intra-day RSD for the replicates

Table 1

Accurate mass measurements of the compounds observed in the HR-ESI-MS and HR-ESI-MS-MS spectra with retention time.

#	Time (min)	Calculated m/z [M+H] ⁺	Precursor ion experimental m/z [M+H] ⁺ /[M+Na] ⁺	Error (mDa)	Elemental composition	Major ions in MS-MS spectra (Key fragment ions)	Collision energy (V)	Identification
1	5.68	251.0914	251.0913	0.1	C ₁₃ H ₁₄ O ₅	233.0802 [*] (100%) 205.0857 (5.4%)	20	Citrinin
2	6.08	321.2060 343.1880	321.2059 343.1881	0.1 −0.1	C ₁₉ H ₂₈ O ₄	285.1836 (18.4%) 225.1624 (46.1) 199.1475 [*] (100%) 173.1317 (67.9%) 159.1159 (29.8%)	20	Monacolin J (lovastatin diol lactone)
3	11.78	423.2741 445.2561	423.2740 445.2565	0.1 −0.4	C ₂₄ H ₃₈ O ₆	303.1925 (22.6%) 285.1833 (81.2%) 267.1739 (34.2%) 225.1626 (38.1%) 199.1469 [*] (100%) 173.1314 (34.5%)	10	Monacolin K acid form
4	13.56	391.2479 413.2298	391.2479 413.2304	0.0 −0.6	C ₂₃ H ₃₄ O ₅	253.1565 (17.5%) 229.1564 (51.1%) 185.1310 [*] (100%) 177.0879 (16.9%) 159.1156 (79.5%)	20	Compactin (Mevastatin)
5	15.29	405.2636 427.2455	405.2639 427.2460	−0.3 −0.5	C ₂₄ H ₃₆ O ₅	285.1835 (26.2%) 267.1728 (18%) 225.1625 (63.8%) 199.1469 [*] (100%) 173.1316 (57.6%) 159.1160 (17.4%)	20	Monacolin K (lovastatin)
6	17.43	279.1128	279.1129	−0.1	C ₁₇ H ₁₄ N ₂ O ₂	262.1087 (5.4%) 248.0932 (17.6%) 156.0437 (26%) 123.0676 [*] (100%) 108.0442 (16.1%)	20	Sudan red G
7	18.26	387.2530 409.2349	387.2529 409.2350	0.1 0.1	C ₂₄ H ₃₄ O ₄	285.1841 (33.4%) 267.1734 (25.7%) 239.1787 (23.3%) 199.1474 [*] (100%) 173.1319 (34.4%) 159.1161 (4.7%)	10	Dehydromonacolin K (dehydrolovastatin)

* base peak

Table 2

Quantitative analysis of RYR commercial raw materials and dietary supplements using UHPLC-QToF-MS.

Sample #	Monacolin K	Monacolin K acid	Mevastatin	Dehydromonacolin K	Lovastatin diol lactone	Citrinin	Sudan red G
Commercial raw materials of RYR from China (mg/g dry weight)							
AU15680	2.19	1.59	DUL	0.09	DUL	ND	ND
AU15681	1.97	1.31	DUL	0.09	DUL	ND	ND
AU15682	2.33	1.41	DUL	0.09	DUL	ND	ND
CR15674	ND	ND	ND	ND	ND	0.02	ND
CR15675	ND	ND	ND	ND	ND	0.01	ND
CR15676	ND	ND	ND	ND	ND	DUL	ND
CR15677	ND	ND	ND	ND	ND	DUL	ND
CR15678	ND	ND	ND	ND	ND	DUL	ND
CR15679	0.68	1.29	DUL	0.07	ND	ND	ND
CR15683	11.95	1.60	0.034	0.81	0.14	ND	ND
CR15684	12.68	1.71	0.030	0.82	0.16	ND	ND
CR15685	11.72	1.21	0.029	0.81	0.14	ND	ND
CR15688	ND	ND	ND	ND	ND	DUL	ND
CR15689	ND	ND	ND	ND	ND	0.01	ND
CR15690	ND	ND	ND	ND	ND	0.08	ND
CR15691	ND	ND	ND	ND	ND	0.01	ND
CR15693	0.204	0.26	DUL	0.1	DUL	ND	ND
CR15694	1.98	1.12	0.01	1.58	0.04	ND	ND
CR15695	0.54	0.62	DUL	0.26	DUL	ND	ND
CR15696	1.92	1.85	DUL	0.86	DUL	ND	ND
CR15697	10.02	2.72	DUL	1.23	0.01	ND	ND
CR15698	24.27	7.56	DUL	1.25	DUL	ND	ND
CR15699	12.74	2.51	0.042	0.31	DUL	ND	ND
CR15700	3.99	5.61	0.028	0.28	0.02	ND	ND
CR15701	17.46	6.32	0.044	1.16	0.05	ND	ND
CR15702	5.08	2.04	0.036	0.35	0.1	ND	ND
CR15703	13.34	5.55	0.042	1.99	0.04	ND	ND
CR15704	19.46	6.23	DUL	1.19	DUL	ND	ND
CR15705	8.73	2.76	0.036	2.15	0.03	ND	ND
CR15706	15.85	4.40	DUL	0.98	DUL	ND	ND
CR15708	1.71	1.66	0.014	1.37	DUL	ND	ND
CR15709	ND	ND	ND	ND	ND	0.01	ND
CR15710	9.41	4.43	DUL	0.99	DUL	ND	ND
CR15711	4.14	1.32	0.030	1.03	0.03	ND	ND
Dietary supplements (mg/ave. wt of capsule or tablet weight)							
DS15715	1.02	0.34	0.01	0.30	0.02	ND	ND
DS15716	0.8	0.19	DUL	0.78	DUL	ND	ND
DS15717	0.11	ND	DUL	0.01	DUL	ND	ND
DS15718	0.12	ND	DUL	0.01	DUL	ND	ND
DS15719	0.11	ND	DUL	0.02	DUL	ND	ND
DS15720	0.03	ND	DUL	0.004	DUL	ND	ND
DS15721	0.44	0.42	DUL	0.18	DUL	ND	ND
DS15722	0.5	ND	DUL	0.06	DUL	ND	ND
DS15723	2.18	0.49	0.02	1.46	0.03	ND	ND
DS15724	0.36	ND	DUL	0.02	DUL	ND	ND
DS15725	1.89	0.39	0.01	0.99	0.02	ND	ND
DS15726	0.84	0.22	DUL	0.32	DUL	ND	ND
DS15727	0.21	0.09	DUL	0.11	DUL	ND	ND
DS15728	2.62	1.72	0.01	1.01	DUL	ND	ND

DUL = Detected under limits of quantification; ND = Not detected

were between 0.5 and 1.7% for compounds **3**, **5**, **7** using the developed UHPLC method. Similarly, the RSD for the day to day replicates were between 0.7 and 2.7%. Two compounds (**2**, **4**) were detected under limits of quantification and two compounds (**1**, **6**) were not detected.

3.3.1.4. Accuracy. The accuracy of the method was determined by spiking an authentic sample (#AU15680) with a known amount of the test compounds. Accuracy of the method was studied using the method of standard addition. Standard compounds (**1–7**) were added to the extract of the RYR powder and the percent recovery was determined at two different levels 50% and 100%. Compounds content was determined and the percent recovery was calculated. The percentage recovery ranged from 96.5 to 101.7%.

3.3.1.5. Stability of the solutions. The sample and standard solutions were prepared according to the proposed method and subjected to

a stability study at room temperature for 24 h. The sample solutions were analyzed at different time intervals up to 24 h. No significant changes were observed in the concentrations of the components over that period of time.

3.3.1.6. Robustness. Robustness tests were carried out during the development stage of the method. The parameters examined were sample amounts, sonication time, different extraction solvents, shaking time, sonication temperature, injection time, centrifugation time, column temperature, flow rate and mobile phase gradient. The extraction time was set at 30 min; increasing the extraction time did not increase the extraction efficiency of the target compounds because the extraction levels of compounds were previously reported to be optimal for this time [1,12,18]. Methanol and 75% ethanol were used as the extraction solvent. Both solvents gave the same levels of extraction efficiency for most of the compounds.

Table 3
Content of MK, MKA, ratios of MK:MKA and Label claim information.

NCNPR code #	Labeled ingredients (Amount/serving size)	Servings recommended/day	Label advises to talk to doctor if taking any medication ^a	Label advises avoiding use with statins or cholesterol medicine	Label informs consumer about symptoms of rhabdomyolysis ^a	Monacolin K (mg/day) ^a	Monacolin K (mg/600 mg of RYR)	Monacolin K acid (mg/600 mg RYR)	Ratio (monacolin K: monacolin K acid)	Dehydro-monacolin K (mg/600 mg RYR)
AU15681	Authentic red yeast rice powder	–	–	–	–	1.2	0.78	1.5:1	0.05	
AU15680	Authentic red yeast rice powder	–	–	–	–	1.32	0.96	1.4:1	0.05	
AU15682	Authentic red yeast rice powder	–	–	–	–	1.38	0.84	1.6:1	0.05	
DS15720	Red yeast rice 600 mg (<i>Monascus purpureus</i>)	4	Yes	No	No	0.12	0.03	ND	–	0.01
DS15717	Red yeast rice powder 600 mg (<i>Monascus purpureus</i>) Policosanol 10 mg	2	Yes	No	Yes	0.22	0.11	ND	–	0.01
DS15727	Organic red yeast rice (<i>Monascus purpureus</i>) 600 mg	2	Yes	No	No	0.43	0.21	0.09	2.3:1	0.11
DS15726	Red yeast rice powder 600 mg (<i>Monascus purpureus</i>) extract	2	No	No	No	1.68	0.84	0.22	3.8:1	0.32
DS15723	Red yeast rice 600 mg (<i>Monascus purpureus</i>)	2	No	No	No	4.35	2.18	0.49	4.5:1	1.46
DS15719	Red yeast rice powder 1200 mg (<i>Monascus purpureus</i>)	2	Yes	No	Yes	0.44	0.11	ND	–	0.02
DS15718	Red yeast rice powder 1200 mg (<i>Monascus purpureus</i>)	2	Yes	No	Yes	0.47	0.12	ND	–	0.01
DS15722	Red yeast rice 1200 mg (1.2 g) (<i>Monascus purpureus</i>) Concentrated (10:1)	2	Yes	Yes	No	0.99	0.25	ND	–	0.03
DS15724	Red yeast rice powder 1200 mg (<i>Monascus purpureus</i>)	1	No	No	No	0.73	0.36	ND	–	0.02
DS15721	Red yeast rice 1200 mg (1.2 g) (<i>Monascus purpureus</i>)	2	Yes	No	No	1.78	0.42	0.44	1.1:1	0.19
DS15716	Red yeast rice complex 1200 mg	2	Yes	No	No	3.20	0.78	0.19	4.1:1	0.78
DS15725	Red yeast rice 1200 mg	2	No	No	Yes	3.79	0.95	0.195	4.9:1	0.49
DS15715	Red yeast rice (<i>Monascus purpureus</i>) [extract] 1200 mg Policosanol (<i>Saccharum officinarum L.</i>) [extracted from sugar cane] 25 mg	1	Yes	No	No	2.03	1.02	0.34	3:1	0.3
DS15728	Red yeast rice (from traditional fermentation of <i>Monascus purpureus</i> WENT) 1200 mg	2	No	No	Yes	10.46	2.62	1.72	1.5:1	1.01

ND, not detected.

^a Amount based on maximum daily intake.

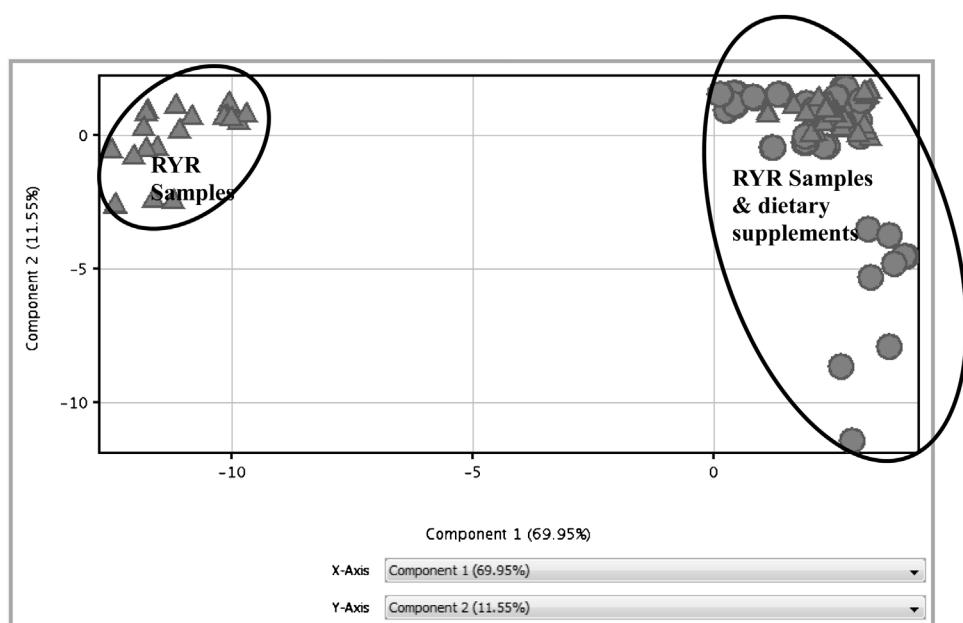


Fig. 3. PCA score plots of RYR samples and dietary supplements.

3.3.1.7. System suitability. System suitability tests are used to ensure reproducibility of the instrument. The test was carried out by injecting 2 μ l of a mixture of standards at least six times. The percent RSD was found to be in the range from 0.10 to 0.33% for all seven compounds, which was acceptable as it is less than 2%.

3.4. Analysis of RYR Samples

To demonstrate the applicability of this method, several commercial products were analyzed. Three authentic samples of RYR, 31 RYR commercial raw materials and 14 dietary supplements were analyzed for the determination of monacolins, citrinin and sudan red G. Once a fingerprint method had been developed through the use of an authenticated sample (#AU15680), other samples of RYR were tested to ensure the method's usefulness. All samples of RYR are listed in the "Experimental Section". The method was validated by testing authenticated sample of RYR. The analyses of compounds **1–7** in the various samples are presented in Table 2 and reflect the substantial diversity among the samples.

The peak of monacolin K (major amount and active ingredient) in RYR samples was identified by comparing the chromatogram and the UV spectrum obtained with the lovastatin standard, which showed a characteristic mountain-like spectrum with three maximum absorptions at (λ_{max}) 230, 237, and 246 nm, respectively. There were 11 other peaks displaying the same or similar UV spectrum of mountain-like peak at λ_{max} 230, 237, and 246 nm as monacolin K.

Three authenticated RYR samples (#AU15680–AU15682) showed monacolin K and monacolin K acid form content in the range from 1.9 to 2.3 mg/g (w/w) and 1.3 to 1.6 mg/g (w/w) dry weight, respectively. Ten of 31 commercial raw material samples of red yeast rice did not show the presence of any monacolins analyzed. From 21 commercial raw material samples of RYR, the amounts of monacolin K and monacolin K acid were in the range from 0.7 to 24.3 mg/g (w/w) and 1.3 to 7.6 mg/g (w/w) dry weight, respectively. In the 14 dietary supplements, the amounts of monacolin K ranged from 0.05 to 4.37 mg/g (w/w).

According to the previous literature [5,7], in properly prepared red yeast rice, the ratios between monacolin K (MK) to monacolin K acid (MKA) vary between 2:1 and 1.5:1. In the present study, the

authentic samples were found to contain in a ratio of MK:MKA that varies from 2:1 to 1.5:1, respectively. The other monacolin components are observed at levels ranging from trace quantities to about 0.02%. For the RYR raw materials contained monacolins, the ratio of MK:MKA ranged from 0.5:1 to 7.5:1. In eight RYR dietary supplements the ratio of MK:MKA ranged from 1.1:1 to 4.9:1 and in 6 products, the ratio could not be shown due to absence of MKA.

All the dietary supplements analyzed contained monacolin K. The quality and contents varied greatly between each product. The levels of MJ, MK, MK acid, compactin and dehydromonacolin K in each sample and product were summarized in Table 2 and Fig. 1S.

Citrinin is a polyketide mycotoxin which was first isolated from *Penicillium citrinum* [23]. The safety of citrinin is controversial because the level at which citrinin causes nephrotoxicity in humans is unknown [24,25]. Citrinin was found in 10 of 31 commercial raw material samples (DUL – 80,000 ng/g (w/w)). Citrinin was not detected in the 14 dietary supplements analyzed.

All RYR samples of red yeast rice were analyzed for the presence of red dye #2 (sudan red G) which might be used as an adulterant due to its low cost. The dye was not detected in any of the samples.

Fourteen dietary supplements (#DS15715–DS15728) were analyzed, in comparison to the authentic samples (#AU15680–82). As shown in Tables 2 and 3, all products showed variations in the total content of compounds **1–7**. All dietary supplements were labeled as containing either 600 mg or 1200 mg of RYR. Twelve products (#DS15716, DS15718–DS15728) were labeled as containing only RYR. Two products (#DS15715 and #DS15717) were labeled as containing the RYR extract along with another botanical (*Saccharum officinarum* L.).

The actual amount of monacolin K consumed daily was calculated based on the recommended daily usage provided on the label. The estimated maximum daily intake (mg/day) was calculated by multiplying the weight monacolin K (mg) by the dilution factor by the suggested maximum daily intake in capsules or tablets/weight (mg) of content in capsules or tablets. For solid dosage forms, the suggested daily use varied from 2 to 4 capsules or 1 to 2 tablets; due to the difference in the composition of these samples, the daily intake also varied (Table 3). While monacolin K content in 600 mg of authentic RYR samples ranged from 1.2 mg to 1.38 mg, amounts of monacolin K in dietary supplements

labeled as containing 600 mg of RYR varied more than 40-fold from 0.03 mg to 2.18 mg. Monacolin K content of dietary supplements labeled as containing 1200 mg RYR varied more than 20-fold from 0.22 mg to 5.23 mg. Total amounts of monacolin K that would be consumed daily using RYR dietary supplements varied almost 10-fold from 0.12 mg/day (#DS15720) to 10.46 mg/day (#DS15728) (Table 3).

3.5. Chemometric analysis

This study also demonstrates that multivariate statistical analysis using UHPLC-QToF-MS provides some useful information in the study of RYR raw materials and dietary supplements. It can be used as a powerful tool to profile and differentiate various phytochemical compositions simultaneously among different samples. The chromatographic region from 5 to 20 min was selected for further studies.

Positive ions with accurate *m/z* values and with a difference corresponding to adduct isotopes or multiply charged species were merged into MFs as a single variable, an entity (a molecular weight, its retention time, and abundance in counts or cps). Entities absent in at least 75% of the samples of a given group were removed to reduce the dimensionality of the data sets prior to PCA. Furthermore, entities were filtered on the basis of *p*-values (*p* < 0.05) calculated for each entity by one-way ANOVA. This ensured the filtration of entities which differed in the respective varieties with statistical significance (98% in this particular case). Compounds that satisfied a fold change cut-off 2.0 (a 4-fold difference) in at least one condition pair were selected for further analysis and differentiation. The extracted entities were mean centered and logarithmically transformed in order to reduce the relative large differences in the respective abundant entities. PCA results were used with 5000 cps threshold data as the results with 1000, 5000 and 10,000 cps thresholds did not show much difference. Molecular formulae were generated to find plant specific biomarkers. PC1 (gives 69.95% of the variability to the original data set) and PC2 (gives 11.55% of the variability to the original data set) together explain 82% of the total variance of the dataset. It indicates a large variation in metabolite composition. The PCA scores plot in Fig. 3 divided into two groups based on the levels and occurrence of monacolins, pigments and citrinin.

4. Conclusions

Chromatographic fingerprinting demonstrated the similarities and differences between various RYR samples analyzed. The tentative identification of many marker and major unknown compounds was performed by the accurate mass and fragmentation patterns. The developed method was validated according to the guidelines of the International Conference on Harmonization (ICH). Consumers of RYR dietary supplements are unable to obtain important information regarding dosage, adverse effects and herb–drug interactions from the dietary supplement labels. Monacolin K content in 600 mg of authentic RYR samples ranged from 1.2 mg to 1.38 mg. Amounts of monacolin K in dietary supplements labeled as containing 600 mg of RYR ranged from 0.03 mg to 2.18 mg. Of the 31 RYR raw materials analyzed, only 21 contained monacolins. The quality of commercial products were also variable in respect to ratio of MK:MKA as well as dehydromonacolin K and citrinin content. The method was found to be simple, precise, accurate, specific and sensitive and can be used for routine quality control of raw materials/dietary supplements and for the quantification of compounds. PCA was applied to the various samples of RYR and dietary supplements as a quality control assessment in order to differentiate their chemical profiles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jpba.2014.07.039>.

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